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(54) Title: MODIFIED GLP-1 RECEPTOR AGONISTS AND THEIR PHARMACOLOGICAL METHODS OF USE

(57) Abstract: This invention relates to modified GLP-1 receptor agonists comprising a GLP-1 receptor agonist linked to a polyethylene glycol polymer having a molecule weight of greater than 30 kD, and related formulations and dosages and methods of administration thereof for therapeutic purposes are provided. More particularly, these modified GLP-1 receptor agonists, compositions and methods are useful in providing a treatment option for those individuals afflicted with a metabolic disorder such as diabetes and prediabetic states such as impaired glucose tolerance, and impaired fasting glucose, by inducing glucose-dependent insulin secretion, without reducing gastrointestinal motility.

MODIFIED GLP-1 RECEPTOR AGONISTS AND THEIR PHARMACOLOGICAL METHODS OF USE

[001] This application claims benefit of U.S. Provisional Application Serial No. 60/408,696, filed September 6, 2002, and U.S. Provisional Application Serial No. 60/439,369, filed January 9, 2003, the contents of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

[002] This invention relates to modified GLP-1 receptor agonists comprising a GLP-1 receptor agonist linked to a polyethylene glycol polymer having a molecular weight of greater than 30 kD, as well as related formulations, dosages and methods of administration thereof for therapeutic purposes. More particularly, these modified GLP-1 receptor agonists, compositions and methods are useful in providing a treatment option for those individuals afflicted with a metabolic disorder such as diabetes, impaired glucose tolerance, or metabolic syndrome, prediabetic states, by inducing glucose-dependent insulin secretion in the absence of the therapeutically limiting side effect of reducing or inhibiting gastrointestinal motility.

BACKGROUND OF THE RELATED ART

[003] Diabetes is characterized by impaired insulin secretion manifesting itself, among other things, by an elevated blood glucose level in the diabetic patient. Underlying defects lead to a classification of diabetes into two major groups: type 1 diabetes, or insulin dependent diabetes mellitus (IDDM), which arises when patients lack β -cells producing insulin in their pancreatic glands, and type 2 diabetes, or non-insulin dependent diabetes mellitus (NIDDM), which occurs in patients with an impaired β -cell insulin secretion and alterations in insulin action.

[004] Type 1 diabetic patients are currently treated with insulin, while type 2 diabetic patients can be treated with agents that stimulate β -cell function or with agents that enhance the tissue sensitivity of the patients towards insulin. Over time almost one-half of type 2 diabetic subjects lose their response to these agents and then must be placed on insulin therapy. The drugs presently used to treat type 2 diabetes are described below.

[005] Alpha-glucosidase inhibitors (e.g., PRECOSE[®], VOGLIBOSE[™], and MIGLITOL[®]) reduce the excursion of postprandial glucose by delaying the absorption of glucose from

the gut. These drugs are safe and provide treatment for mild to moderately affected diabetic subjects. However, gastrointestinal side effects have been reported in the literature and limit their effectiveness.

[006] Insulin sensitizers are drugs that enhance the body's response to insulin. Thiazolidinediones such as Avandia™ (rosiglitazone) and Actos™ activate the Peroxisome proliferator-activated receptor (PPAR) gamma and modulate the activity of a set of genes that have not been well described. Rezulin™ (troglitazone), the first drug in this class, was withdrawn because elevated liver enzyme levels and drug induced hepatotoxicity. These hepatic effects do not appear to be a significant problem in patients using Avandia™ and Actos™. Even so, liver enzyme testing is recommended every 2 months in the first year of therapy and periodically thereafter. Avandia™ and Actos™ treatments are associated with fluid retention, edema and weight gain. Avandia™ is not indicated for use with insulin because of concern about congestive heart failure.

[007] Insulin secretagogues such as sulfonylureas (SFUs) and the non-sulfonylureas (e.g., Nateglinide and Pepaglinide) act through the ATP-dependent K⁺ channel to cause glucose independent insulin secretion. These drugs are standard therapy for type 2 diabetics that have mild to moderate fasting glycemia. The SFUs have limitations that include a potential for inducing hypoglycemia, weight gain, and high primary and secondary failure rates. Ten to 20% of initially treated patients fail to show a significant treatment effect (primary failure). Secondary failure is demonstrated by an additional 20-30% loss of treatment effect after six months on an SFU. Insulin treatment is required in 50% of the SFU responders after 5-7 years of therapy (Schein, et al., Diabetes Res. Clin. Pract. 6:533-543, 1989). Nateglinide and Pepaglinide are short-acting drugs that need to be taken three times a day. They are used only for the control of post-prandial glucose and not for control of fasting glucose.

[008] GLUCOPHAGE™ (metformin HCl) is a biguanide that lowers blood glucose by decreasing hepatic glucose output and increasing peripheral glucose uptake and utilization. The drug is effective at lowering blood glucose in mildly and moderately affected subjects, and does not have the side effects of weight gain or the potential to induce hypoglycemia. However, GLUCOPHAGE™ has a number of side effects including gastrointestinal disturbances and lactic acidosis. GLUCOPHAGE™ is contraindicated in diabetics over the age of 70 and in subjects with impairment in renal or liver function. Finally, GLUCOPHAGE™ has the same primary and secondary failure rates as the SFUs.

[009] Insulin treatment is instituted after diet, exercise, and oral medications have failed to adequately control blood glucose. This treatment has the drawbacks that it is an injectable, can produce hypoglycemia, and causes weight gain. The possibility of inducing hypoglycemia with insulin limits the extent that hyperglycemia can be controlled.

[010] Because of the problems with current treatments, new therapies to treat type 2 diabetes are needed. In particular, new treatments to retain normal (glucose-dependent) insulin secretion are needed. Such new drugs should have the following characteristics: dependent on glucose for promoting insulin secretion (i.e., produce insulin secretion only in the presence of elevated blood glucose); low primary and secondary failure rates; and preserve islet cell function. The strategy to develop the new therapy disclosed herein is based on the cyclic adenosine monophosphate (cAMP) signaling mechanism and its effects on insulin secretion.

[011] Glucose is a major regulator of the insulin secretion process. Elevation of this sugar promotes the closure of the K⁺ channels following the elevation of ATP. Closure of the K⁺ channels causes cell depolarization and subsequent opening of Ca⁺⁺ channels, which in turn leads to exocytosis of insulin granules. Little, if any, effects on insulin secretion occurs in the absence of low glucose concentrations (Weinhaus, et al., Diabetes 47:1426-1435, 1998). Secretagogues like GLP-1 utilize the cAMP system to regulate insulin secretion through this glucose-dependent mechanism (Komatsu, et al., Diabetes 46:1928-1938, 1997; Filippson, et al., Diabetes 50:1959-1969, 2001; Drucker, Endocrinology 142:521-527, 2001). Insulin secretagogues via the elevation of cAMP is also able to enhance insulin synthesis in addition to insulin release (Skoglund, et al., Diabetes 49:1156-1164, 2000; Borboni, et al., Endocrinology 140:5530-5537, 1999).

[012] GLP-1 (glucagon-like peptide 1) is released from the intestinal L-cell after a meal and functions as an incretin hormone (i.e., it potentiates glucose-induced insulin release from the pancreatic β -cell). It is a 37-amino acid peptide that is differentially expressed by the glucagon gene, depending upon tissue type. The clinical data that support the beneficial effect of raising cAMP levels in β -cells have been collected with GLP-1. Infusions of GLP-1 in poorly controlled type 2 diabetics normalized their fasting blood glucose levels (Gutniak, et al., New Eng. J. Med. 326:1316-1322, 1992) and with longer infusions improved the β -cell function to those of normal subjects (Rachman, et al., Diabetes 45:1524-1530, 1996). A recent report has shown that GLP-1 improves the ability of β -cells to respond to glucose in subjects with impaired glucose tolerance (Byrne, et al., Diabetes 47:1259-1265, 1998). All of these effects, however, are short-lived because of the short half-life of the peptide.

[013] In addition to the short half-life of the GLP-1 peptide, GLP-1 reduces gut motility (see, e.g., Kieffer and Habener, Endocrine Reviews 20:876-913, 1999 and Drucker, Gastroenterology 122:531-544, 2002), which in turn results in significant gastrointestinal side effects, such as nausea and vomiting. Such gastrointestinal side effects have also been demonstrated with GLP-1 agonists, such as NN-2211 (Agerso, et al., Diabetologia 45:195-202, 2002) and Exendin-4 (Amylin abstract, American Diabetes Association meeting, 2001). The reduction in gut motility that causes the gastrointestinal side effects has been studied in rodent models (Lotti, et al., Life Sci. 39:1631-1638, 1986). The inhibitory effects of GLP-1 on the gastrointestinal motility and secretion have been shown to be at least partially mediated by the central nervous system (Imeryuz, et al., Am. J. Physiol 273:G920-G927, 1997). Systemically injected GLP-1 gains access to the brain from the periphery by simple diffusion (Kastin, et al., J. Mol. Neurosci. 18:7-14, 2002).

[014] Therefore, there exists a need for improved peptides that have the glucose-dependent insulin secretagogue activity of GLP-1, but without the side effects which limits GLP-1 based treatments.

SUMMARY OF THE INVENTION

[015] This invention relates to modified GLP-1 receptor agonists comprising a GLP-1 receptor agonist linked to a polyethylene glycol polymer having a molecular weight of greater than 30 kD, and which retains its ability to agonize the GLP-1 receptor. These modified GLP-1 agonists are effective in the treatment of metabolic disorders, such as diabetes or impaired glucose tolerance, a prediabetic state. Moreover, the modified GLP-1 agonists of this invention are capable of treating metabolic disorders without inhibiting gastrointestinal motility, thereby producing fewer gastrointestinal side effects, such as nausea and vomiting and allowing higher more effective doses to be administered.

[016] In particular, one aspect of the invention is a polypeptide that functions as a GLP-1 receptor agonist. Examples of GLP-1 receptor agonists include, but are not limited to, the polypeptides shown in Figure 1 and include those polypeptides selected from the group consisting of SEQ ID NOs: 1-10, and fragments and variants of the polypeptide that function as an agonist of the GLP-1 receptor at substantially the same level as the polypeptides of the listed SEQ ID NOs: 1-10 (collectively, polypeptides of this invention).

[017] Another embodiment of the invention is polynucleotides that encode for the GLP-1 receptor agonist polypeptides, and the attendant vectors and host cells necessary to recombinantly express the polypeptides of this invention.

[018] Another aspect of this invention is a modified GLP-1 receptor agonist comprising one of the polypeptides of this invention, or fragments or variants thereof, that function as an agonist of the GLP-1 receptor at substantially the same level as the polypeptides of the invention that has been modified by linking to it a polyethylene glycol polymer having a molecular weight of greater than 30 kD (collectively, "modified polypeptides of this invention"). Examples of modified GLP-1 receptor agonists include, but are not limited to, the modified polypeptides shown in Figure 2 and include those polypeptides selected from the group consisting of SEQ ID NOs: 13-14 and 16-31.

[019] The invention is also directed to methods of making the GLP-1 agonist polypeptides of this invention, both recombinant and synthetic, and methods of making the modified GLP-1 agonist polypeptides of this invention.

[020] Also disclosed are methods of treating diabetes and/or other diseases or conditions in a mammal without causing gastrointestinal side effects, comprising administering a therapeutically effective amount of any of the modified polypeptides of the present invention to said mammal.

BRIEF DESCRIPTION OF THE DRAWING

[021] FIG. 1 depicts amino acid sequences of polypeptides identified as SEQ ID NOs:1-10 which are examples of GLP-1 receptor agonists, the polypeptides of the invention.

[022] FIG. 2 depicts amino acid sequences of polypeptides of SEQ ID NOs:13-14 and 16-31, which are examples of modified GLP-1 agonists, the modified polypeptides of this invention.

[023] FIG. 3A is a line graph showing that fatty acid-modified GLP-1 reduced gastrointestinal motility.

[024] FIG. 3B is a line graph showing that GLP-1 modified with a 22 kD PEG reduced gastrointestinal motility, whereas GLP-1 modified with a 43 kD PEG did not reduce gastrointestinal motility.

[025] FIG. 4 is a line graph showing that a GLP-1 agonist of this invention (SEQ ID NO: 26) modified with a 22 kD or 30 kD PEG reduced gastrointestinal motility, whereas the same GLP-1 agonist peptide modified with a 43 kD PEG did not reduce gastrointestinal motility.

[026] FIG. 5 is a bar graph showing that a GLP-1 agonist of this invention (SEQ ID NO: 26) modified with a 43 kD PEG will reduce gastrointestinal motility if injected ICV.

DESCRIPTION OF THE INVENTION

[027] This invention relates to modified GLP-1 receptor agonists comprising a GLP-1 receptor agonist linked to a polyethylene glycol (PEG) polymer having a molecular weight of greater than 30 kD, and methods of administration thereof for therapeutic purposes are provided. More particularly, these modified GLP-1 receptor agonists and compositions function *in vivo* as GLP-1 receptor agonists in the prevention and/or treatment of such diseases or conditions as diabetes, hyperglycemia, impaired glucose tolerance, impaired fasting glucose, and obesity by inducing glucose-dependent insulin secretion, without reducing gastrointestinal motility.

[028] Peptides having GLP-1 receptor agonist activity have been identified, and include, for example, GLP-1 (7-36), GLP-1 (7-37), Exendin-4, and other GLP-1 analogs (see, e.g., WO 98/43658, WO 00/15224, WO 00/16797, WO 01/98331, U.S. Patent No. 5,545,618; U.S. Patent No. 5,118,666; and U.S. Patent Application Serial No. 60/395,738; the references of which are incorporated herein in their entirety).

[029] The stacking alignment below shows the primary structural relationships between some of the known GLP-1 agonist peptides:

		<u>SEQ</u> <u>ID NO.</u>
GLP-1 (7-36)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH ₂	1
GLP-1 (7-37)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	2
Exendin-4 (G27)	HGEGTFTSDL SKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH ₂ HSQGTFTSDYAKYLDARRAKEFIAWLVKGR-NH ₂	3 4

[030] As used herein, the single letter abbreviation for a particular amino acid, its corresponding amino acid, and three letter abbreviation are as follows: A, alanine (ala); C, cysteine (cys); D, aspartic acid (asp); E, glutamic acid (glu); F, phenylalanine (phe); G, glycine (gly); H, histidine (his); I, isoleucine (ile); K, lycine (lys); L, leucine (leu); M, methionine (met); N, asparagine (asn); P, proline (pro); Q, glutamine (gln); R, arginine (arg); S, serine (ser); T, threonine (thr); V, valine (val); W, tryptophan (trp); and Y, tyrosine (tyr).

[031] These polypeptides play a role in glucose homeostasis, and in particular, these peptides function as GLP-1 receptor agonists by lowering plasma glucose concentrations. Given GLP-1's role in promoting glucose-regulated insulin secretion in the pancreas, GLP-1 receptor agonists are potentially valuable in the treatment of metabolic disorders and other diseases. To date, however, GLP-1 receptor agonists have had significant

side effects; namely a reduction in gastrointestinal motility, which in turn can lead to nausea and vomiting.

[032] It is well known in the art that PEGylation of a drug such as small molecules, peptides, or proteins can improve plasma half-life, physical solubility and stability, and resistance to protease degradation as well as reduce immunogenicity. Furthermore, it has been suggested in the art that PEGylation can reduce the extent of adverse side effects by reducing the trough to peak levels of the drug that result from sustained plasma concentrations. However, it was not known in the art that PEGylation could limit access of a drug to a certain tissue.

[033] Surprisingly, modification of a peptide or protein with a particular size or structure of a polymer, such as PEG, can selectively affect tissue distribution of the administered modified peptide or protein. For example, modification of GLP-1 agonists with a linear 22 kD PEG does not increase the therapeutic index (glucose-lowering vs. gut motility) as compared to unmodified GLP-1 agonists and C16-fatty acid modified agonists. Modification with a linear 30 kD PEG modestly improved the therapeutic index, whereas modification with a branched 43 kD PEG greatly reduced CNS-mediated gut motility. Interestingly, when injected directly into the brain, the 43 kD-PEGylated GLP-1 agonist was able to induce gut motility. Thus, PEG size and structure are major determinants of such a selective process.

[034] Despite the many advantages attributed to PEGylation, one significant disadvantage is the interference of the bulky PEG in the interaction between the peptide and its receptor, resulting in a reduction of functional activity. Utilizing GLP-1 and glucagon as models, a “twisted helix” model was developed, and based on this model, positions located within the C-terminal of the peptide were selected for PEGylation. These positions were predicted to be on the opposing side of the peptide-receptor interaction.

[035] The inventors herein have found that modifying the GLP-1 receptor agonists by linking a polyethylene glycol polymer having a molecular weight of greater than 30 kD to the GLP-1 receptor agonist will inhibit the reduction in gastrointestinal motility typically associated with GLP-1 receptor agonists. Without being bound to theory, the inventors herein believe that increasing the size of the GLP-1 receptor agonist using PEGylation technology prevents the GLP-1 receptor agonist from crossing the blood-brain-barrier and thus, accessing the central nervous system. As a result, the GLP-1 agonists’ ability to cause gastrointestinal side effects (which are likely to be mediated by the central nervous system) is reduced. The PEGylated GLP-1 receptor agonist, however, still has access to

the pancreas and thus, lowers blood glucose, the desired activity for treating type 2 diabetes.

[036] GLP-1 Receptor Agonists:

The polypeptides of this invention are GLP-1 receptor agonists and are determined as such by their ability to activate the GLP-1 receptor. The GLP-1 receptor agonist activates the GLP-1 receptor in one or more *in vitro* or *in vivo* assays for GLP-1 receptor activation. Examples of such assays include, but are not limited to, *in vitro* assays for induction of cAMP in RINm5F cells, *in vitro* assays for induction of insulin secretion from pancreatic β -cells, *in vivo* assays for reduction in plasma glucose levels, and *in vivo* assays for elevation in plasma insulin levels as described in the specific examples below.

[037] Examples of the GLP-1 receptor agonists include, but are not limited to, the polypeptides selected from the group consisting of SEQ ID NOS: 1-10 and fragments, derivatives, variants and analogs thereof, that function as an agonist of the GLP-1 receptor at substantially the same level as the polypeptides of the listed SEQ ID NOS: 1-10

[038] GLP-1 receptor agonists, the polypeptides of the present invention, may be naturally-occurring polypeptides, recombinant polypeptides, or synthetic peptides.

[039] Fragments, derivatives, variants and analogs of GLP-1 receptor agonists:

Fragment, derivative, variant, and analog polypeptides retain substantially the same biological function or activity as, for example, polypeptides shown in SEQ ID NOS: 1-10. "Substantially the same biological function or activity" each means that degree of biological activity that is within about 30% to about 100% (*i.e.*, 30, 40, 50, 60, 70, 80, 90, or 100%) or more of that biological activity demonstrated by the polypeptide to which it is being compared when the biological activity of each polypeptide is determined by the same procedure.

[040] A fragment is less than a full-length polypeptide, such as the polypeptides shown in SEQ ID NOS: 1-10, which retains substantially similar functional activity, as shown in the *in vitro* and *in vivo* models disclosed herein.

[041] Derivatives include polypeptides that have been chemically modified to provide an additional structure and/or function. For example, a fatty acid can be added to a polypeptide to improve its half-life. Fusion polypeptides which confer targeting specificity or an additional activity also can be constructed, as described in more detail below.

[042] Derivatives can be modified by either a natural process, such as posttranslational processing, or by chemical modification techniques, both of which are well known in the art. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a variant may contain one or more different types of modifications. Polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching.

[043] Other chemical modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formulation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristylation, oxidation, pegylation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination (see, e.g., T. E. Creighton, PROTEINS, STRUCTURE AND MOLECULAR PROPERTIES, 2nd ed., W.H. Freeman and Company, New York (1993); POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, ed., Academic Press, New York, pgs. 1-14 (1983); Seifter, et al., Meth. Enzymol 182:626-46, 1990; Rattan, et al., Ann. N.Y. Acad. Sci. 663:48-62, 1992).

[044] Derivatives also include mature polypeptides that have been fused with another polypeptide, for example, human serum albumin, to improve their pharmacokinetic profile. Fusion of two polypeptides can be accomplished by any means known to one skilled in the art. For example, a DNA encoding human serum albumin and a DNA sequence encoding a polypeptide of the invention can be cloned into any mammalian expression vector known to one skilled in the art. Location of a polypeptide of the invention N-terminal to the other polypeptide is preferred, because it appears that a free N-terminal histidine is required for GLP-1 receptor activity (Kawa, Endocrinology 124(49):1768-73, 1989). The resulting recombinant fusion protein can then be expressed by transforming a suitable cell line, such as HKB or CHO, with the vector and expressing the fusion protein.

[045] Variants of polypeptides of the invention include polypeptides having one or more amino acid sequence changes with respect to the amino acid sequences shown in SEQ ID NOS: 1-10. Variants also can have amino acids joined to each other by modified

peptide bonds (i.e., peptide isosteres) and may contain amino acids other than the 20 naturally occurring amino acids.

[046] Preferably, variants contain one or more conservative amino acid substitutions (i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions), preferably at nonessential amino acid residues. A “nonessential” amino acid residue is a residue that can be altered from a wild-type sequence of a protein without altering its biological activity, whereas an “essential” amino acid residue is required for biological activity. A conservative amino acid substitution is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Non-conservative substitutions would not be made for conserved amino acid residues or for amino acid residues residing within a conserved protein domain.

[047] Variants also include polypeptides that differ in amino acid sequence due to mutagenesis. Variants that function as GLP-1 receptor agonists can be identified by screening combinatorial libraries of mutants, for example, mutants of polypeptides with conservative substitutions at one or more positions (i.e., at 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 positions) can be screened for GLP-1 receptor agonist activity using methods well known in the art, and described in Examples 3, 4, 5, and 6.

[048] An analog includes a propolypeptide, which includes an amino acid sequence of a polypeptide of the invention. Active polypeptides of the invention can be cleaved from the additional amino acids in the propolypeptide molecule by natural, *in vivo* processes, or by procedures well known in the art, such as by enzymatic or chemical cleavage.

[049] Polynucleotides of this Invention and Methods of Producing the Polypeptides

Any polynucleotide sequence that encodes a polypeptide of the invention can be used to express the polypeptide. Polynucleotides can consist only of a coding sequence for a polypeptide or can include additional coding and/or non-coding sequences.

[050] Polynucleotide sequences encoding a polypeptide of the invention can be synthesized in whole or in part using chemical methods well known in the art (see, e.g.,

Caruthers, et al., Nucl. Acids Res. Symp. Ser. 215–23, 1980; Horn, et al., Nucl. Acids Res. Symp. Ser 225-32, 1980). The polynucleotide that encodes the polypeptide can then be cloned into an expression vector to express the polypeptide, or into a cloning vector to propagate the polynucleotide.

[051] As will be understood by those of skill in the art, it may be advantageous to produce the polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of polypeptide expression or to produce an RNA transcript having desirable properties, such as a half-life, which is longer than that of a transcript generated from the naturally occurring sequence.

[052] The nucleotide sequences disclosed herein can be engineered using methods generally known in the art to alter the polypeptide-encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the polypeptide or mRNA product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides can be used to engineer the nucleotide sequences. For example, site-directed mutagenesis can be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

[053] The present invention also includes cloning and expression vectors comprising one or more nucleotide sequences encoding a polypeptide of the invention. The nucleotide sequence can be inserted in a forward or reverse orientation. A DNA sequence may be inserted into a vector by a variety of procedures. In general, a DNA sequence is inserted into an appropriate restriction endonuclease site by procedures known in the art and described in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., (Cold Spring Harbor, N.Y., 1989). Such procedures and others are deemed to be within the scope of those skilled in the art.

[054] Examples of cloning vectors include, but are not limited to pBR322, pUC18, pUC19, pSport, and pCRII.

[055] An expression vector may further comprise regulatory sequences, including, for example, a promoter, operably linked to the coding sequence. Large numbers of suitable expression vectors and promoters are known to those of skill in the art and are commercially available. The following expression vectors are provided by way of example. Bacterial expression vectors include, but are not limited to, pQE70, pQE60, pQE-9 (Qiagen); pBS, phagescript, psiX174, pBluescript SK, pBsKS, pNH8a, pNH16a,

pNH18a, pNH46a (Stratagene); and pTRC99A, pKK223-3, pKK233-3, pDR540, PRIT5 (Pharmacia). Eukaryotic expression vectors include, but are not limited to, pWLneo, pSV2cat, pOG44, pXT1, pSG (Stratagene); and pSVK3, pBPV, pMSG, PSVL (Pharmacia). However, any other cloning or expression vector may be used as long as it is replicable and viable in the desired host. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) expression vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of an appropriate vector and promoter is well within the level of ordinary skill in the art.

[056] An expression vector also may contain a ribosome binding site for translation initiation, a transcription terminator, and appropriate sequences for amplifying expression. Expression vectors may contain a gene to provide a phenotypic trait for selection of transformed host cells, such as dihydrofolate reductase or neomycin resistance for a eukaryotic cell culture, or such as tetracycline or ampicillin resistance for culture in *E. coli*.

[057] In one embodiment, a library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A library of variants can be produced, for example, by enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential variant amino acid sequences is expressible as individual polypeptides or, alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of sequences therein.

[058] There are a variety of methods that can be used to produce libraries of potential variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential analog sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, Tetrahedron 39:3, 1983; Itakura, et al., Annu. Rev. Biochem. 53:323, 1984; Itakura, et al., Science 198:1056, 1984; Ike, et al., Nucleic Acid Res. 11:477, 1983).

[059] Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for

rapid screening of the gene libraries generated by the combinatorial mutagenesis of polypeptides of the invention. The most widely used techniques, which are amenable to high through-put analysis for screening large gene libraries, typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique that enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify the desired variants.

[060] The present invention also provides host cells containing the above-described vectors. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell. Alternatively, the host cell can be a prokaryotic cell, such as a bacterial cell.

[061] Host cells can be genetically engineered (transduced, transformed, or transfected) with cloning or expression vectors of the invention. The vector may be, for example, in the form of a plasmid, a viral particle, or a phage. Engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters or selecting transformants. The selection of appropriate culture conditions, such as temperature and pH, are well within the skill of the ordinarily skilled artisan.

[062] As representative examples of appropriate hosts, include, but are not limited to, bacterial cells such as *E. coli*, *Salmonella typhimurium*, or *Streptomyces*; fungal cells such as yeast; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; or mammalian cells such as CHO, COS, or Bowes melanoma. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

[063] Introduction of the construct into the host cell can be effected, for example, by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, et al., BASIC METHODS IN MOLECULAR BIOLOGY, 1986). Constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence.

[064] Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with

prokaryotic and eukaryotic hosts are described above and in Sambrook, et al., (MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., Cold Spring Harbor, N.Y., 1989).

[065] Transcription of a DNA encoding polypeptides of the present invention by higher eukaryotes can be increased by inserting an enhancer sequence into the expression vector. Enhancers are *cis*-acting elements of DNA, usually from about 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell (e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene) and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation, initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics (e.g., stabilization or simplified purification of expressed recombinant product).

[066] After transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is derepressed by appropriate means (e.g., temperature shift or chemical induction), and cells are cultured for an additional period. Cells are typically harvested by centrifugation and disrupted by physical or chemical means. The resulting crude extract is retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

[067] Various mammalian cell culture systems also can be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts (Gluzman, Cell 23:175, 1981), and other cell lines capable of expressing a compatible vector, for example, C127, 3T3, CHO, HeLa, and HBK cell lines.

[068] Polypeptides of the present invention may be recovered and purified from recombinant cell cultures by methods well known in the art, including ammonium sulfate

or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography, and lectin chromatography. High performance liquid chromatography (HPLC) can be employed as a final purification step.

[069] Polypeptides of the invention can be conveniently isolated. A purified polypeptide is at least about 70% pure, that is, the isolated polypeptide is substantially free of cellular material and has less than about 30% (by dry weight) of non-polypeptide material. Preferably, the preparations are 85% through 99% (i.e., 85, 87, 89, 91, 93, 95, 96, 97, 98, and 99%) pure. Purity of the preparations can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis, mass spectroscopy, and liquid chromatography.

[070] Depending upon the host employed in a recombinant production procedure, polypeptides of the invention may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

[071] Alternatively, polypeptides of the invention can be produced using chemical methods to synthesize its amino acid sequence, such as by direct peptide synthesis using solid-phase techniques (see, e.g., Merrifield, J. Am. Chem. Soc. 85:2149–2154, 1963; Roberge, et al., Science 269:202–204, 1995). Polypeptide synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer).

Optionally, fragments of a polypeptide can be separately synthesized and combined using chemical methods to produce a full-length molecule.

[072] A newly synthesized polypeptide can be substantially purified by preparative high performance liquid chromatography (see, e.g., Creighton, Proteins: Structures And Molecular Principles, WH Freeman and Co., New York, N.Y., 1983). The composition of a synthetic polypeptide of the present invention can be confirmed by amino acid analysis or sequencing by, for example, the Edman degradation procedure (see, Creighton, *supra*). Additionally, any portion of the amino acid sequence of the polypeptide can be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins to produce a variant polypeptide or a fusion polypeptide.

[073] Modified GLP-1 Receptor Agonists and Methods of Production

The modified GLP-1 receptor agonists of the present invention comprise a GLP-1 receptor agonist, or a fragment, derivative, variant or analog thereof, which is linked to a polyethylene glycol (PEG) polymer having a molecular weight of greater than 30 kD.

[074] Suitable PEG polymers typically are commercially available or may be made by techniques well known to those skilled in the art. The PEG polymer has a molecular weight of greater than 30 kD, preferably a molecular weight of greater than 30 kD, more preferably greater than 40 kD, and still more preferably having a branched structure, such as for example, a 43 kD branched PEG-peptide (Shearwater 2001 catalog # 2D3X0T01, mPEG2-MAL).

[075] The attachment of a PEG on an intact peptide can be accomplished by attaching the PEG on the opposite side of the peptide surface that interacts with the receptor. Preferably, the attachment of PEG will occur on the GLP-1 agonist, numbered in accordance with GLP-1 (7-37), at positions 22-28 and 30-31, as well as at positions past the C terminus; namely positions 32-37. More preferably, attachment of the PEG will occur on the GLP-1 agonist, numbered in accordance with GLP-1 (7-37), at positions 24, 28, 30 and 31, as well as at positions past the C terminus; namely at positions 32, 34, 36 and 37. Still more preferably, attachment of the PEG will occur on the GLP-1 agonist, numbered in accordance with GLP-1 (7-37), at the C terminus; namely position 31.

[076] There are several strategies for coupling PEG to peptides (see, e.g., Veronese, Biomaterials 22:405-417, 2001), all of which are incorporated herein by reference in their entirety. Those skilled in the art, therefore, will be able to utilize such well-known techniques for linking the PEG polymer to the GLP-1 receptor agonists described herein.

[077] Briefly, cysteine PEGylation is one method for site-specific PEGylation, and it is often utilized if a peptide has few or no free cysteines. In the case of the native GLP-1 (7-37), for example, there are no cysteine residues. Accordingly, PEGylation of native GLP-1 (7-37) or any GLP-1 agonist having no cysteine residues can be accomplished by introducing a unique cysteine mutation at one of the specific positions on the native GLP-1 (7-37) or the GLP-1 agonist identified above and then reacting the resulting derivative with a cysteine-specific PEGylation reagent, such as PEG-maleimide.

[078] However, it may be necessary to mutate the GLP-1 agonist of this invention in order to allow for site-specific PEGylation. For example, if a GLP-1 agonist contains cysteine residues, these will need to be substituted with a conservative amino acids in order to ensure site specific PEGylation. In addition, rigid linkers, including but not limited to, "GGS" (SEQ ID NO: 32), "GGSGGS" (SEQ ID NO: 33), and "PPPS" (SEQ ID NO: 34),

may be added to the C-terminus of the GLP-1 agonist, but before the site of PEG attachment (i.e., a unique cysteine residue).

[079] Examples of the modified GLP-1 receptor agonists of this invention include, but are not limited to, the polypeptides selected from the group consisting of SEQ ID NOs: 13-14 and 16-31. The most preferred modified GLP-1 receptor agonist of this invention is SEQ ID NO: 26.

[080] The polypeptides of the present invention may be employed in treatment diabetes, including both type 1 and type 2 diabetes (non-insulin dependent diabetes mellitus). Such treatment may also delay the onset of diabetes and diabetic complications. The polypeptides may be used to prevent subjects with impaired glucose tolerance from proceeding to develop type 2 diabetes. Other diseases and conditions that may be treated or prevented using compounds of the invention in methods of the invention include: Maturity-Onset Diabetes of the Young (MODY) (Herman, et al., *Diabetes* 43:40, 1994); Latent Autoimmune Diabetes Adult (LADA) (Zimmet, et al., *Diabetes Med.* 11:299, 1994); impaired glucose tolerance (IGT) (Expert Committee on Classification of Diabetes Mellitus, *Diabetes Care* 22 (Supp. 1):S5, 1999); impaired fasting glucose (IFG) (Charles, et al., *Diabetes* 40:796, 1991); gestational diabetes (Metzger, *Diabetes*, 40:197, 1991); and metabolic syndrome X.

[081] The polypeptides of the present invention may also be effective in such disorders as obesity, and in the treatment of atherosclerotic disease, hyperlipidemia, hypercholesterolemia, low HDL levels, hypertension, cardiovascular disease (including atherosclerosis, coronary heart disease, coronary artery disease, and hypertension), cerebrovascular disease and peripheral vessel disease.

[082] The compounds of the present invention may also be useful for treating physiological disorders related to, for example, cell differentiation to produce lipid accumulating cells, regulation of insulin sensitivity and blood glucose levels, which are involved in, for example, abnormal pancreatic β -cell function, insulin secreting tumors and/or autoimmune hypoglycemia due to autoantibodies to insulin, autoantibodies to the insulin receptor, or autoantibodies that are stimulatory to pancreatic β -cells), macrophage differentiation which leads to the formation of atherosclerotic plaques, inflammatory response, carcinogenesis, hyperplasia, adipocyte gene expression, adipocyte differentiation, reduction in the pancreatic β -cell mass, insulin secretion, tissue sensitivity to insulin, liposarcoma cell growth, polycystic ovarian disease, chronic anovulation, hyperandrogenism, progesterone production, steroidogenesis, redox potential and oxidative stress in cells, nitric oxide synthase (NOS) production, increased gamma

glutamyl transpeptidase, catalase, plasma triglycerides, HDL, and LDL cholesterol levels, and the like.

[083] Polypeptides of the invention may also be used in methods of the invention to treat secondary causes of diabetes (Expert Committee on Classification of Diabetes Mellitus, Diabetes Care 22 (Supp. 1):S5, 1999). Such secondary causes include glucocorticoid excess, growth hormone excess, pheochromocytoma, and drug-induced diabetes. Drugs that may induce diabetes include, but are not limited to, pyriminil, nicotinic acid, glucocorticoids, phenytoin, thyroid hormone, β -adrenergic agents, α -interferon and drugs used to treat HIV infection.

[084] The polypeptides of the present invention may be used alone or in combination with additional therapies and/or compounds known to those skilled in the art in the treatment of diabetes and related disorders. Alternatively, the methods and compounds described herein may be used, partially or completely, in combination therapy.

[085] The polypeptides of the invention may also be administered in combination with other known therapies for the treatment of diabetes, including PPAR agonists, sulfonylurea drugs, non-sulfonylurea secretagogues, α -glucosidase inhibitors, insulin sensitizers, insulin secretagogues, hepatic glucose output lowering compounds, insulin and anti-obesity drugs. Such therapies may be administered prior to, concurrently with or following administration of the polypeptides of the invention. Insulin includes both long and short acting forms and formulations of insulin. PPAR agonist may include agonists of any of the PPAR subunits or combinations thereof. For example, PPAR agonist may include agonists of PPAR- α , PPAR- γ , PPAR- δ or any combination of two or three of the subunits of PPAR. PPAR agonists include, for example, rosiglitazone and pioglitazone. Sulfonylurea drugs include, for example, glyburide, glimepiride, chlorpropamide, and glipizide. α -glucosidase inhibitors that may be useful in treating diabetes when administered with a polypeptide of the invention include acarbose, miglitol and voglibose. Insulin sensitizers that may be useful in treating diabetes include thiazolidinediones and non-thiazolidinediones. Hepatic glucose output lowering compounds that may be useful in treating diabetes when administered with a polypeptide of the invention include metformin, such as Glucophage and Glucophage XR. Insulin secretagogues that may be useful in treating diabetes when administered with a polypeptide of the invention include sulfonylurea and non-sulfonylurea drugs: GIP, secretin, nateglinide, meglitinide, repaglinide, glibenclamide, glimepiride, chlorpropamide, glipizide. In one embodiment of the invention, polypeptides of the invention are used in combination with insulin secretagogues to increase the sensitivity of pancreatic β -cells to the insulin secretagogue.

[086] Polypeptides of the invention may also be used in methods of the invention in combination with anti-obesity drugs. Anti-obesity drugs include β -3 agonists, CB-1 antagonists, appetite suppressants, such as, for example, sibutramine (Meridia), and lipase inhibitors, such as, for example, orlistat (Xenical).

[087] Polypeptides of the invention may also be used in methods of the invention in combination with drugs commonly used to treat lipid disorders in diabetic patients. Such drugs include, but are not limited to, HMG-CoA reductase inhibitors, nicotinic acid, bile acid sequestrants, and fibrin acid derivatives. Polypeptides of the invention may also be used in combination with anti-hypertensive drugs, such as, for example, β -blockers and ACE inhibitors.

[088] Such co-therapies may be administered in any combination of two or more drugs (e.g., a compound of the invention in combination with an insulin sensitizer and an anti-obesity drug). Such co-therapies may be administered in the form of pharmaceutical compositions, as described above.

[089] As used herein, various terms are defined below.

[090] When introducing elements of the present invention or the preferred embodiment(s) thereof, the articles "a," "an," "the," and "said" are intended to mean that there are one or more of the elements. The terms "comprising," "including," and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

[091] The term "subject" as used herein includes mammals (e.g., humans and animals).

[092] The term "treatment" includes any process, action, application, therapy, or the like, wherein a subject, including a human being, is provided medical aid with the object of improving the subject's condition, directly or indirectly, or slowing the progression of a condition or disorder in the subject.

[093] The term "combination therapy" or "co-therapy" means the administration of two or more therapeutic agents to treat a diabetic condition and/or disorder. Such administration encompasses co-administration of two or more therapeutic agents in a substantially simultaneous manner, such as in a single capsule having a fixed ratio of active ingredients or in multiple, separate capsules for each inhibitor agent. In addition, such administration encompasses use of each type of therapeutic agent in a sequential manner.

[094] The phrase "therapeutically effective" means the amount of each agent administered that will achieve the goal of improvement in a diabetic condition or disorder severity, while avoiding or minimizing adverse side effects associated with the given therapeutic treatment.

[095] The term "pharmaceutically acceptable" means that the subject item is appropriate for use in a pharmaceutical product.

[096] Based on well known assays used to determine the efficacy for treatment of conditions identified above in mammals, and by comparison of these results with the results of known medicaments that are used to treat these conditions, the effective dosage of the polypeptides of this invention can readily be determined for treatment of each desired indication. The amount of the active ingredient (e.g., polypeptides) to be administered in the treatment of one of these conditions can vary widely according to such considerations as the particular compound and dosage unit employed, the mode of administration, the period of treatment, the age and sex of the patient treated, and the nature and extent of the condition treated.

[097] The total amount of the active ingredient to be administered may generally range from about 0.0001 mg/kg to about 200 mg/kg, and preferably from about 0.01 mg/kg to about 200 mg/kg body weight per day. A unit dosage may contain from about 0.05 mg to about 1500 mg of active ingredient, and may be administered one or more times per day. The daily dosage for administration by injection, including intravenous, intramuscular, subcutaneous, and parenteral injections, and use of infusion techniques may be from about 0.01 to about 200 mg/kg. The daily rectal dosage regimen may be from 0.01 to 200 mg/kg of total body weight. The transdermal concentration may be that required to maintain a daily dose of from 0.01 to 200 mg/kg.

[098] Of course, the specific initial and continuing dosage regimen for each patient will vary according to the nature and severity of the condition as determined by the attending diagnostician, the activity of the specific polypeptide employed, the age of the patient, the diet of the patient, time of administration, route of administration, rate of excretion of the drug, drug combinations, and the like. The desired mode of treatment and number of doses of a polypeptide of the present invention may be ascertained by those skilled in the art using conventional treatment tests.

[099] The polypeptides of this invention may be utilized to achieve the desired pharmacological effect by administration to a patient in need thereof in an appropriately formulated pharmaceutical composition. A patient, for the purpose of this invention, is a

mammal, including a human, in need of treatment for a particular condition or disease. Therefore, the present invention includes pharmaceutical compositions which are comprised of a pharmaceutically acceptable carrier and a therapeutically effective amount of a polypeptide. A pharmaceutically acceptable carrier is any carrier which is relatively non-toxic and innocuous to a patient at concentrations consistent with effective activity of the active ingredient so that any side effects ascribable to the carrier do not vitiate the beneficial effects of the active ingredient. A therapeutically effective amount of a polypeptide is that amount which produces a result or exerts an influence on the particular condition being treated. The polypeptides described herein may be administered with a pharmaceutically-acceptable carrier using any effective conventional dosage unit forms, including, for example, immediate and timed release preparations, orally, parenterally, topically, or the like.

[100] For oral administration, the polypeptides may be formulated into solid or liquid preparations such as, for example, capsules, pills, tablets, troches, lozenges, melts, powders, solutions, suspensions, or emulsions, and may be prepared according to methods known to the art for the manufacture of pharmaceutical compositions. The solid unit dosage forms may be a capsule which can be of the ordinary hard- or soft-shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers such as lactose, sucrose, calcium phosphate, and corn starch.

[101] In another embodiment, the polypeptides of this invention may be tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch, or gelatin; disintegrating agents intended to assist the break-up and dissolution of the tablet following administration such as potato starch, alginic acid, corn starch, and guar gum; lubricants intended to improve the flow of tablet granulation and to prevent the adhesion of tablet material to the surfaces of the tablet dies and punches, for example, talc, stearic acid, or magnesium, calcium or zinc stearate; dyes; coloring agents; and flavoring agents intended to enhance the aesthetic qualities of the tablets and make them more acceptable to the patient. Suitable excipients for use in oral liquid dosage forms include diluents such as water and alcohols, for example, ethanol, benzyl alcohol, and polyethylene alcohols, either with or without the addition of a pharmaceutically acceptable surfactant, suspending agent, or emulsifying agent. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance tablets, pills or capsules may be coated with shellac, sugar or both.

[102] Dispersible powders and granules are suitable for the preparation of an aqueous suspension. They provide the active ingredient in admixture with a dispersing or wetting agent, a suspending agent, and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example, those sweetening, flavoring and coloring agents described above, may also be present.

[103] The pharmaceutical compositions of this invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil such as liquid paraffin or a mixture of vegetable oils. Suitable emulsifying agents may be (1) naturally occurring gums such as gum acacia and gum tragacanth, (2) naturally occurring phosphatides such as soy bean and lecithin, (3) esters or partial esters derived from fatty acids and hexitol anhydrides, for example, sorbitan monooleate, and (4) condensation products of said partial esters with ethylene oxide, for example, polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening and flavoring agents.

[104] Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil such as, for example, arachis oil, olive oil, sesame oil, or coconut oil; or in a mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent such as, for example, beeswax, hard paraffin, or cetyl alcohol. The suspensions may also contain one or more preservatives, for example, ethyl or *n*-propyl p-hydroxybenzoate; one or more coloring agents; one or more flavoring agents; and one or more sweetening agents such as sucrose or saccharin.

[105] Syrups and elixirs may be formulated with sweetening agents such as, for example, glycerol, propylene glycol, sorbitol, or sucrose. Such formulations may also contain a demulcent, and preservative, flavoring and coloring agents.

[106] The polypeptides of this invention may also be administered parenterally, that is, subcutaneously, intravenously, intramuscularly, or interperitoneally, as injectable dosages of the compound in a physiologically acceptable diluent with a pharmaceutical carrier which may be a sterile liquid or mixture of liquids such as water, saline, aqueous dextrose and related sugar solutions; an alcohol such as ethanol, isopropanol, or hexadecyl alcohol; glycols such as propylene glycol or polyethylene glycol; glycerol ketals such as 2,2-dimethyl-1,1-dioxolane-4-methanol, ethers such as poly(ethyleneglycol) 400; an oil; a fatty acid; a fatty acid ester or glyceride; or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant such as a soap or a detergent, suspending agent such as pectin, carbomers, methycellulose,

hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agent and other pharmaceutical adjuvants.

[107] Illustrative of oils which can be used in the parenteral formulations of this invention are those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, sesame oil, cottonseed oil, corn oil, olive oil, petrolatum, and mineral oil. Suitable fatty acids include oleic acid, stearic acid, and isostearic acid. Suitable fatty acid esters are, for example, ethyl oleate and isopropyl myristate. Suitable soaps include fatty alkali metal, ammonium, and triethanolamine salts and suitable detergents include cationic detergents, for example, dimethyl dialkyl ammonium halides, alkyl pyridinium halides, and alkylamine acetates; anionic detergents, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates; nonionic detergents, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylenepolypropylene copolymers; and amphoteric detergents, for example, alkyl-beta-aminopropionates, and 2-alkylimidazoline quaternary ammonium salts, as well as mixtures.

[108] The parenteral compositions of this invention may typically contain from about 0.5% to about 25% by weight of the active ingredient in solution. Preservatives and buffers may also be used advantageously. In order to minimize or eliminate irritation at the site of injection, such compositions may contain a non-ionic surfactant having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulation ranges from about 5% to about 15% by weight. The surfactant can be a single component having the above HLB or can be a mixture of two or more components having the desired HLB.

[109] Illustrative of surfactants used in parenteral formulations are the class of polyethylene sorbitan fatty acid esters, for example, sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

[110] The pharmaceutical compositions may be in the form of sterile injectable aqueous suspensions. Such suspensions may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents such as, for example, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents which may be a naturally occurring phosphatide such as lecithin, a condensation product of an alkylene oxide with a fatty acid, for example, polyoxyethylene stearate, a condensation product of ethylene oxide with a long chain aliphatic alcohol, for example,

heptadecaethyleneoxycetanol, a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol such as polyoxyethylene sorbitol monooleate, or a condensation product of an ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride, for example polyoxyethylene sorbitan monooleate.

[111] The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent. Diluents and solvents that may be employed are, for example, water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile fixed oils are conventionally employed as solvents or suspending media. For this purpose, any bland, fixed oil may be employed including synthetic mono or diglycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables.

[112] A composition of the invention may also be administered in the form of suppositories for rectal administration of the drug. These compositions may be prepared by mixing the drug (e.g., polypeptide) with a suitable non-irritation excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such material are, for example, cocoa butter and polyethylene glycol.

[113] Another formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the compounds of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art (see, e.g., U.S. Patent No. 5,023,252, incorporated herein by reference). Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents.

[114] It may be desirable or necessary to introduce the pharmaceutical composition to the patient via a mechanical delivery device. The construction and use of mechanical delivery devices for the delivery of pharmaceutical agents is well known in the art. For example, direct techniques for administering a drug directly to the brain usually involve placement of a drug delivery catheter into the patient's ventricular system to bypass the blood-brain barrier. One such implantable delivery system, used for the transport of agents to specific anatomical regions of the body, is described in U.S. Patent No. 5,011,472, incorporated herein by reference.

[115] The compositions of the invention may also contain other conventional pharmaceutically acceptable compounding ingredients, generally referred to as carriers or

diluents, as necessary or desired. Any of the compositions of this invention may be preserved by the addition of an antioxidant such as ascorbic acid or by other suitable preservatives. Conventional procedures for preparing such compositions in appropriate dosage forms can be utilized.

[116] Commonly used pharmaceutical ingredients which may be used as appropriate to formulate the composition for its intended route of administration include: acidifying agents, for example, but are not limited to, acetic acid, citric acid, fumaric acid, hydrochloric acid, nitric acid; and alkalinizing agents such as, but are not limited to, ammonia solution, ammonium carbonate, diethanolamine, monoethanolamine, potassium hydroxide, sodium borate, sodium carbonate, sodium hydroxide, triethanolamine, trolamine.

[117] Other pharmaceutical ingredients include, for example, but are not limited to, adsorbents (e.g., powdered cellulose and activated charcoal); aerosol propellants (e.g., carbon dioxide, CCl_2F_2 , $\text{F}_2\text{ClC-CClF}_2$ and CClF_3); air displacement agents (e.g., nitrogen and argon); antifungal preservatives (e.g., benzoic acid, butylparaben, ethylparaben, methylparaben, propylparaben, sodium benzoate); antimicrobial preservatives (e.g., benzalkonium chloride, benzethonium chloride, benzyl alcohol, cetylpyridinium chloride, chlorobutanol, phenol, phenylethyl alcohol, phenylmercuric nitrate and thimerosal); antioxidants (e.g., ascorbic acid, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorus acid, monothioglycerol, propyl gallate, sodium ascorbate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite); binding materials (e.g., block polymers, natural and synthetic rubber, polyacrylates, polyurethanes, silicones and styrene-butadiene copolymers); buffering agents (e.g., potassium metaphosphate, potassium phosphate monobasic, sodium acetate, sodium citrate anhydrous and sodium citrate dihydrate); carrying agents (e.g., acacia syrup, aromatic syrup, aromatic elixir, cherry syrup, cocoa syrup, orange syrup, syrup, corn oil, mineral oil, peanut oil, sesame oil, bacteriostatic sodium chloride injection and bacteriostatic water for injection); chelating agents (e.g., edetate disodium and edetic acid); colorants (e.g., FD&C Red No. 3, FD&C Red No. 20, FD&C Yellow No. 6, FD&C Blue No. 2, D&C Green No. 5, D&C Orange No. 5, D&C Red No. 8, caramel and ferric oxide red); clarifying agents (e.g., bentonite); emulsifying agents (but are not limited to, acacia, cetomacrogol, cetyl alcohol, glyceryl monostearate, lecithin, sorbitan monooleate, polyethylene 50 stearate); encapsulating agents (e.g., gelatin and cellulose acetate phthalate); flavorants (e.g., anise oil, cinnamon oil, cocoa, menthol, orange oil, peppermint oil and vanillin); humectants (e.g., glycerin, propylene glycol and sorbitol);

levigating agents (e.g., mineral oil and glycerin); oils (e.g., arachis oil, mineral oil, olive oil, peanut oil, sesame oil and vegetable oil); ointment bases (e.g., lanolin, hydrophilic ointment, polyethylene glycol ointment, petrolatum, hydrophilic petrolatum, white ointment, yellow ointment, and rose water ointment); penetration enhancers (transdermal delivery) (e.g., monohydroxy or polyhydroxy alcohols, saturated or unsaturated fatty alcohols, saturated or unsaturated fatty esters, saturated or unsaturated dicarboxylic acids, essential oils, phosphatidyl derivatives, cephalin, terpenes, amides, ethers, ketones and ureas); plasticizers (e.g., diethyl phthalate and glycerin); solvents (e.g., alcohol, corn oil, cottonseed oil, glycerin, isopropyl alcohol, mineral oil, oleic acid, peanut oil, purified water, water for injection, sterile water for injection and sterile water for irrigation); stiffening agents (e.g., cetyl alcohol, cetyl esters wax, microcrystalline wax, paraffin, stearyl alcohol, white wax and yellow wax); suppository bases (e.g., cocoa butter and polyethylene glycols (mixtures)); surfactants (e.g., benzalkonium chloride, nonoxynol 10, oxtoxynol 9, polysorbate 80, sodium lauryl sulfate and sorbitan monopalmitate); suspending agents (e.g., agar, bentonite, carbomers, carboxymethylcellulose sodium, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, kaolin, methylcellulose, tragacanth and veegum); sweetening e.g., aspartame, dextrose, glycerin, mannitol, propylene glycol, saccharin sodium, sorbitol and sucrose); tablet anti-adherents (e.g., magnesium stearate and talc); tablet binders (e.g., acacia, alginic acid, carboxymethylcellulose sodium, compressible sugar, ethylcellulose, gelatin, liquid glucose, methylcellulose, povidone and pregelatinized starch); tablet and capsule diluents (e.g., dibasic calcium phosphate, kaolin, lactose, mannitol, microcrystalline cellulose, powdered cellulose, precipitated calcium carbonate, sodium carbonate, sodium phosphate, sorbitol and starch); tablet coating agents (e.g., liquid glucose, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose, ethylcellulose, cellulose acetate phthalate and shellac); tablet direct compression excipients (e.g., dibasic calcium phosphate); tablet disintegrants (e.g., alginic acid, carboxymethylcellulose calcium, microcrystalline cellulose, polacrillin potassium, sodium alginate, sodium starch glycollate and starch); tablet glidants (e.g., colloidal silica, corn starch and talc); tablet lubricants (e.g., calcium stearate, magnesium stearate, mineral oil, stearic acid and zinc stearate); tablet/capsule opaquants (e.g., titanium dioxide); tablet polishing agents (e.g., carnuba wax and white wax); thickening agents (e.g., beeswax, cetyl alcohol and paraffin); tonicity agents (e.g., dextrose and sodium chloride); viscosity increasing agents (e.g., alginic acid, bentonite, carbomers, carboxymethylcellulose sodium, methylcellulose, povidone, sodium alginate and tragacanth); and wetting agents

(e.g., heptadecaethylene oxycetanol, lecithins, polyethylene sorbitol monooleate, polyoxyethylene sorbitol monooleate, and polyoxyethylene stearate).

[118] The polypeptides described herein may be administered as the sole pharmaceutical agent or in combination with one or more other pharmaceutical agents where the combination causes no unacceptable adverse effects. For example, the polypeptides of this invention can be combined with known anti-obesity, or with known antidiabetic or other indication agents, and the like, as well as with admixtures and combinations thereof.

[119] The polypeptides described herein may also be utilized, in free base form or in compositions, in research and diagnostics, or as analytical reference standards, and the like. Therefore, the present invention includes compositions which are comprised of an inert carrier and an effective amount of a compound identified by the methods described herein, or a salt or ester thereof. An inert carrier is any material which does not interact with the compound to be carried and which lends support, means of conveyance, bulk, traceable material, and the like to the compound to be carried. An effective amount of compound is that amount which produces a result or exerts an influence on the particular procedure being performed.

[120] Polypeptides are known to undergo hydrolysis, deamidation, oxidation, racemization and isomerization in aqueous and non-aqueous environment. Degradation such as hydrolysis, deamidation or oxidation can readily detected by capillary electrophoresis. Enzymatic degradation notwithstanding, polypeptides having a prolonged plasma half-life, or biological resident time, should, at minimum, be stable in aqueous solution. It is essential that polypeptide exhibits less than 10% degradation over a period of one day at body temperature. It is still more preferable that the polypeptide exhibits less than 5% degradation over a period of one day at body temperature. Because of the life time treatment in chronic diabetic patient, much preferably these therapeutic agents are convenient to administer, furthermore infrequently if by parenteral route. Stability (i.e., less than a few percent of degradation) over a period of weeks at body temperature will allow less frequent dosing. Stability in the magnitude of years at refrigeration temperature will allow the manufacturer to present a liquid formulation, thus avoid the inconvenience of reconstitution. Additionally, stability in organic solvent would provide polypeptide be formulated into novel dosage forms such as implant.

[121] Formulations suitable for subcutaneous, intravenous, intramuscular, and the like; suitable pharmaceutical carriers; and techniques for formulation and administration may

be prepared by any of the methods well known in the art (see, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 20th edition, 2000).

[122] The following examples are presented to illustrate the invention described herein, but should not be construed as limiting the scope of the invention in any way.

[123] Capsule Formulation

A capsule formula is prepared from:

Polypeptide of this invention	10 mg
Starch	109 mg
Magnesium stearate	1 mg

The components are blended, passed through an appropriate mesh sieve, and filled into hard gelatin capsules.

[124] Tablet Formulation

A tablet is prepared from:

Polypeptide of this invention	25 mg
Cellulose, microcrystalline	200 mg
Colloidal silicon dioxide	10 mg
Stearic acid	5.0 mg

The ingredients are mixed and compressed to form tablets. Appropriate aqueous and non-aqueous coatings may be applied to increase palatability, improve elegance and stability or delay absorption.

[125] Sterile IV Solution

A mg/mL solution of the desired compound of this invention is made using sterile, injectable water, and the pH is adjusted if necessary. The solution is diluted for administration with sterile 5% dextrose and is administered as an IV infusion.

[126] Intramuscular suspension

The following intramuscular suspension is prepared:

Polypeptide of this invention	50 µg/mL
Sodium carboxymethylcellulose	5 mg/mL

TWEEN 80	4 mg/mL
Sodium chloride	9 mg/mL
Benzyl alcohol	9 mg/mL

The suspension is administered intramuscularly.

[127] Hard Shell Capsules

A large number of unit capsules are prepared by filling standard two-piece hard galantine capsules each with powdered active ingredient, 150 mg of lactose, 50 mg of cellulose, and 6 mg of magnesium stearate.

[128] Soft Gelatin Capsules

A mixture of active ingredient in a digestible oil such as soybean oil, cottonseed oil, or olive oil is prepared and injected by means of a positive displacement pump into molten gelatin to form soft gelatin capsules containing the active ingredient. The capsules are washed and dried. The active ingredient can be dissolved in a mixture of polyethylene glycol, glycerin and sorbitol to prepare a water miscible medicine mix.

[129] Immediate Release Tablets/Capsules

These are solid oral dosage forms made by conventional and novel processes. These units are taken orally without water for immediate dissolution and delivery of the medication. The active ingredient is mixed in a liquid containing ingredient such as sugar, gelatin, pectin, and sweeteners. These liquids are solidified into solid tablets or caplets by freeze drying and solid state extraction techniques. The drug compounds may be compressed with viscoelastic and thermoelastic sugars and polymers or effervescent components to produce porous matrices intended for immediate release, without the need of water.

[130] It should be apparent to one of ordinary skill in the art that changes and modifications can be made to this invention without departing from the spirit or scope of the invention as it is set forth herein.

EXAMPLES

[131] In order that this invention may be better understood, the following examples are set forth. These examples are for the purpose of illustration only, and are not to be construed as limiting the scope of the invention in any manner. All publications mentioned herein are incorporated by reference in their entirety.

[132] Example 1. Peptide Synthesis Methodology.

The following general procedure was followed to synthesize some of the polypeptides of the invention. Peptide synthesis was carried out by the FMOC/t-Butyl strategy (Peptide Synthesis Protocols, Volume 35, Michael W. Pennington & Ben M. Dunn, 1994) under continuous flow conditions using Rapp-Polymer PEG-Polystyrene resins (Rapp-Polymer, Tubingen, Germany). At the completion of synthesis, peptides were cleaved from the resin and de-protected using TFA/DTT/H₂O/Triisopropyl silane (88/5/5/2). Peptides were precipitated from the cleavage cocktail using cold diethyl ether. The precipitate was washed three times with the cold ether and then dissolved in 5% acetic acid prior to lyophilization. Peptide identity was confirmed by reversed-phase chromatography on a YMC-Pack ODS-AQ column (YMC, Inc., Wilmington, NC) on a Waters ALLIANCE® system (Waters Corporation, Milford, MA) using water/acetonitrile with 3% TFA as a gradient from 0% to 100% acetonitrile, and by MALDI mass spectrometry on a VOYAGER DE™ MALDI Mass Spectrometer, (model 5-2386-00, PerSeptive BioSystems, Framingham, MA). Matrix buffer (50/50 dH₂O/acetonitrile with 3% TFA) peptide sample was added to Matrix buffer 1/1. Those peptides not meeting the purity criteria of >95% were purified by reversed-phase chromatography on a Waters Delta Prep 4000 HPLC system (Waters Corporation, Milford, MA).

[133] Table 1 contains some selected polypeptides made according to the Peptide Synthesis protocols discussed above.

[134]

Table 1

Peptide	Peptide Sequence	SEQ ID NO:
GLP-1(7-36) amide	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH ₂	1
GLP-1(7-37)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	2
Exendin 4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS-NH ₂	3
G27	HSQGTFTSDYAKYLDARRAKEFIAWLVKGR-NH ₂	4
G51	HSQGTFTSDYAKYLDARRAKEFIAWLVKGRG	5
G1	HSQGTFTSDYSKYLDSRRAQDFVQWLVKGR-NH ₂	7
G5	HSQGTFTSDYSKYLEGQAAKEFIAWLVKGR-NH ₂	8
G55	HSQGTFTSDYARYLDARRAKEFIAWLVKGR-NH ₂	9
G56	HSQGTFTSDYAAYLDARRAKEFIAWLVKGR-NH ₂	10

[135] Example 2. Peptide PEGylation

PEGylation may be performed by any method known to those skilled in the art. However, in this instance, PEGylation was performed using two different methods to introduce a unique cysteine mutation into the peptide or at the C-terminus of the peptide, followed by PEGylating the cysteine via a stable thioether linkage between the sulphydryl of the peptide and maleimide group of the methoxy-PEG-maleimide reagent (Inhale/Shearwater). It is preferable to introduce a unique cysteine at the C-terminus of the peptide.

[136] In the first method, a 2-fold molar excess of mPEG-mal (MW 22 kD, 30 kD, and 43 kD) reagent was added to 1 mg peptide and dissolved in reaction buffer at pH 6 (0.1 M Na phosphate/ 0.1 M NaCl/ 0.1 M EDTA). After 0.5 hour at room temperature, the reaction was stopped with 2-fold molar excess of DTT to mPEG-mal. The peptide-PEG-mal reaction mixture was applied to a cation exchange column to remove residual PEG reagents followed by gel filtration column to remove residual free peptide. The purity, mass, and number of PEGylated sites were determined by SDS-PAGE and MALDI-TOF mass spectrometry.

[137] In the second method, a 10-fold molar excess of mPEG-mal (MW ~22 kD or 43 kD) reagent was added to 50 µM peptide dissolved in reaction buffer at pH 6 (0.1 M Na phosphate/ 0.1 M NaCl/ 0.1 M EDTA). After 0.5 hour at room temperature, the reaction was stopped with 2-fold molar excess of cysteine over mPEG-mal. The crude peptide-PEG-mal reaction mixtures were assayed *in vitro* without further purification.

[138] Table 2 contains some selected polypeptides made according to the PEGylation technology discussed herein. Note that the underlined amino acid represents the location that the PEG polymer is attached to the peptide.

[139]

Table 2

Peptide	Peptide Sequence	SEQ ID NO:
G71	HSQGTFTSDYAKYLDARRACEFIAWLVKGRG	11
G72	HSQGTFTSDYAKYLDARRAKCFIAWLVKGRG	12
G73	HSQGTFTSDYAKYLDARRAKEFICWLVKGRG	13
G74	HSQGTFTSDYAKYLDARRAKEFIAWLVCGRG	14
G75	HSQGTFTSDYAKYLDARRAKEFIAWLVKCRG	15
G76	HSQGTFTSDYAKYLDARRAKEFIAWLVKCGC	16
G77	HSQGTFTSDYAKYLDARRAKEFIAWLVKGRC	17
G78	HSQGTFTSDYAKYLDARRAKEFIAWLVKGRGC	18
G79	HSQGTFTSDYAKYLDARRAKEFIAWLVKGRGGSC	19
G80	HSQGTFTSDYAKYLDARRAKEFIAWLVKGRGPPPC	20
G81	HSQGTFTSDYAKYLDARRAKEFIAWLVKGRGGSGGSC	21
G82	HSQGTFTSDYARYLDARRAKEFIAWLVRGRG	22
G83	HSQGTFTSDYARYLDARRAREFIKWLVRGRG	23
G84	HSQGTFTSDYARYLDARRAREFIAWLVKGRG	24
G85	HSQGTFTSDYARYLDARRAREFIAWLVRGRGK	25
G185	HSQGTFTSDYARYLDARRAREFIKWLVRGRGC	26

[140] Example 3. Measurement of Peptide Signaling Through GLP1 Receptor Using Cyclic AMP Scintillation Proximity (SPA) Assay

For the modified GLP-1 receptor agonists of this invention, “activation” of the GLP-1 receptor in a cAMP scintillation proximity assay is induction of a maximum activity that is at least 50%, more preferably at least 70%, still more preferably at least 80%, and still more preferably at least 90% of the maximal activity induced by the native GLP-1(7-36)-amide. The EC50 value for a modified GLP-1 receptor agonist of this invention is between 0.1 and 1000 nM. “EC50” is defined herein as the concentration of a polypeptide of this invention at which 50% of the maximal activity is achieved.

[141] RINm5F cells were plated in 96-well plates (Costar) at 1.5×10^5 cells/well and grown at 37°C for 24 hours in RPMI 1640, 5% FBS, antibiotic/antimycotic (Gibco BRL). The media was removed and the cells were washed twice with PBS. The cells were incubated with peptide concentrations ranging from 1×10^{-12} to 1×10^{-5} M in HEPES-PBS containing 1% BSA and 100 µM IBMX for 15 minutes at 37°C. The incubation buffer was removed, and the cells lysed in lysis reagent provided with the cAMP Scintillation Proximity Assay (SPA) direct screening assay system (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The amount of cAMP (in pmol) present in the lysates was determined following instructions provided with this kit. The amount of cAMP (in pmol) produced at each concentration of peptide was plotted and analyzed by nonlinear regression using Prism software to determine the EC50 for each peptide.

[142] The results of this assay with controls (identified with an asterisk) and representative polypeptides of this invention are shown in the table below.

[143]

Table 3

Peptide	22 kD PEG			43 kDa PEG			
	EC50	unmodified	PEG	fold-shift	EC50	unmodified	PEG
G71 *	27.3	346.1	14.3		33.6	358.6	10.7
G72 *	88.0	476.0	5.2		83.6	411.3	4.9
G73	40.4	144.5	4.2		36.0	294.7	8.2
G74	36.0	114.7	3.7		53.8	337.4	7.2
G75 *	48.3	275.8	7.6		53.7	388.4	8.0
G76	20.4	106.6	5.5		26.0	383.0	14.8
G77	26.1	49.7	2.0		22.4	236.1	11.0
G78	15.7	69.5	3.3		20.1	91.6	4.9
G79	19.6	60.2	3.0		16.6	66.9	4.0
G80	14.9	53.2	4.1				
G81	20.5	77.6	4.2		16.9	49.6	3.0

[144] Example 4. Insulin Secretion from Dispersed Rat Islet Cells

Increase of insulin secretion from dispersed rat islet cells, in this assay, is an increase of at least 1.5 fold. The modified GLP-1 agonist of this invention increases insulin secretion from dispersed islet cells by at least about 1.5 fold to about 10 fold (i.e., 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, or 10-fold).

[145] Islets of Langerhans were isolated from SD rats (200-250 g) through a digestion procedure using collagenase. Dispersed islet cells were prepared through treatment with trypsin, seeded into 96 V-bottom plates and pelleted. Cells were cultured overnight in media with and without peptides of the invention. Media was aspirated and the cells were pre-incubated with Krebs-Ringer-HEPES buffer containing 3 mM glucose for 30 minutes at 37°C. Pre-incubation buffer was removed and cells were stimulated with Krebs-Ringer-HEPES buffer containing the appropriate glucose concentration (e.g., 8 mM), with and without peptides for an appropriate time at 37°C. In some studies, an appropriate concentration of GLP-1 also is included. A portion of supernatant was removed and its insulin content was measured by SPA. The results were expressed as fold over control (FOC). At a concentration of 50 nM, the modified GLP-1 agonist having an amino acid sequence shown in SEQ ID NO: 26 with a 43 kD PEG linked to the C terminus increased insulin secretion from dispersed islet cells approximately 3 fold.

[146] Example 5. Measuring Increases in Plasma Insulin Levels during IVGTT in Fasted Wistar Rats

An increase in plasma insulin levels in this assay is an increase of at least about 2-fold. Preferably, the modified GLP-1 receptor agonist of this invention increases insulin secretion in rats as measured by an increase in plasma insulin levels during *in vivo* glucose tolerance testing in fasted Wistar rats by about 2-fold to about 5-fold, more preferably by about 2-fold to about 10 fold, and still more preferably by about 2-fold to about 20-fold (i.e., 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20-fold).

[147] Male Wistar rats will be fasted overnight and then anesthetized with isoflurane gas. The rats will be given a tail vein injection of 0.4 g/kg of glucose plus either vehicle (0.9% saline + 1% albumin) or 1 nmol/kg GLP-1 (positive control) or 1 nmol/kg of the polypeptides of this invention. The rats will then be eye-bled one minute later and the plasma assayed for insulin using an ELISA Kit (Alpco Diagnostics, Windham, NH).

[148] Example 6. Effect of PEGylated peptides of this invention on Intraperitoneal Glucose Tolerance in Mice

A decrease in blood glucose levels as measured by this assay is a decrease of at least about 10%. Preferably, the modified GLP-1 receptor agonist of the invention decreases blood glucose levels in mice as measured by intraperitoneal glucose tolerance testing in rats or mice by about 10% to about 60%, more preferably by about 10% to about 80%, still more preferably by about 10% to about 100% (i.e., 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100%).

[149] The *in vivo* activity of the modified peptides (PEGylated) of the invention when administered subcutaneously was examined in mice. Mice fasted overnight were given a subcutaneous injection of control or modified peptide (100 µg/kg). Three hours later, basal blood glucose was measured, and the rats were given 2 g/kg of glucose intraperitoneally. Blood glucose was measured again after 30 and 60 minutes.

[150] The representative modified peptides of the invention significantly reduced blood glucose levels relative to the vehicle following the IPGTT, with 36%-54% reduction in the glucose AUC ("area under curve"). This demonstrates that modified peptides have prolonged glucose lowering activity *in vivo*. In addition to the glucose lowering activity of the modified peptides of the invention, it also indicates prolonged peptide half-life *in vivo*. Unmodified GLP-1 has a very short half-life *in vivo* (< 10 minUTES). The ability of the modified peptides of the invention to lower blood glucose 3 hours following peptide

administration is a clear indication that the peptide is present in the circulation at this time point and hence has prolonged half-life relative to unmodified GLP-1.

[151] Example 7: Measurement of Gastrointestinal Motility in Mice by Subcutaneous Injection

Modified GLP-1 receptor agonists of this invention have a therapeutic index of at least 5-fold. Preferably, the modified GLP-1 receptor agonist of this invention has a therapeutic index of about 5-fold to about 10-fold, more preferably by about 5-fold to about 20 fold, still more preferably by about 5-fold to about 50-fold, even more preferable by about 5-fold to 100-fold, and most preferably by about 5-fold to 200-fold. The therapeutic index is the minimum concentration of peptide (i.e., agonist) required to reduce gut motility by at least 20% divided by minimum concentration required to reduce blood glucose AUC by at least 20% (see Example 6).

[152] Gastrointestinal motility in mice was tested using representative modified GLP-1 agonists of this invention (GLP-1 agonist linked to a 43 kD PEG, GLP-1 agonist linked to a 30 kD PEG, GLP-1 agonist peptides linked to 22 kD PEG, and GLP-1 agonist linked to a fatty acid). Gastrointestinal motility was measured as follows: Male Balb/c mice were either fasted overnight first and then given peptide (3-100 µg/kg) or vehicle by subcutaneous injection, or they were given peptide or vehicle first and then fasted overnight, depending on the time interval between dosing and the measurement of motility. At the appropriate time after dosing, the mice were given a charcoal meal by oral gavage, and then euthanized by cervical dislocation five minutes later. The small intestine was dissected out and the length of the intestine measured as well as the length the charcoal traveled past the pyloric sphincter. The % traveled was calculated by dividing the distance the charcoal traveled by the total length of the small intestine and multiplying by 100.

[153] In one example, fatty acid-modified -GLP1 (R^{34} -GLP-1(7-37) with K26 modified by γ -L-glutamoyl($N\alpha$ -hexadecanoyl)) was given to overnight fasted mice 3 hours prior to the charcoal meal. Fatty acid-GLP1 dose-dependently reduced gastrointestinal motility (Figure 3A).

[154] In a second example, GLP-1 linked to a 22 kD PEG was given to overnight fasted mice 3 hours prior to the charcoal meal. GLP-1 linked to a 22 kD PEG also dose-dependently reduced gastrointestinal motility (Figure 3B).

[155] In a third example, GLP-1 linked to a 43 kD PEG was given to overnight fasted mice 3 hours prior to the charcoal meal. GLP-1 linked to a 43 kD PEG showed no significant effect on gastrointestinal motility (Figure 3B).

[156] In a forth example, GLP-1 agonist linked to a 22 kD PEG was given to overnight fasted mice 3 hours prior to the charcoal meal. This GLP-1 agonist linked to a 22 kD PEG also dose-dependently reduced gastrointestinal motility (Figure 4).

[157] In a fifth example, GLP-1 agonist linked to a 30 kD PEG was given to overnight fasted mice 3 hours prior to the charcoal meal. This GLP-1 agonist linked to a 30 kD PEG also dose-dependently reduced gastrointestinal motility (Figure 4).

[158] In a sixth example, the representative modified GLP-1 agonist of this invention (GLP-1 agonist linked to a 43 kD PEG) was given to overnight fasted mice 3 hours prior to the charcoal meal. The representative modified GLP-1 agonist of this invention showed no significant effect on gastrointestinal motility (Figure 4).

[159] Example 8: Measurement of Gastrointestinal Motility in Mice by ICV Injection

Stainless steel guide cannulas that were aimed at the brain third ventricle were implanted to male Wistar rats (275-350 g) anesthetized with isoflurane anesthesia. The single 21G cannula was aimed at the brain third ventricle using a stereotaxic instrument and the following coordinates: -2.2 mm posterior from bregma and -7.5 mm ventral to dura. The cannula was secured to the skull with jeweler's screws and dental cement. One week after surgery, cannula placement was tested by infusion of 1 μ l Angiotensin II at 10 ng/ μ l concentration. Animals drinking 5 ml or more of water in one-hour period were retained for the study. On the day of the gastric motility experiment, overnight fasted rats were infused into the third ventricle with vehicle (PBS), 0.5 μ g/rat of GLP-1 (7-36) amide peptide, or 20 μ g/rat SEQ ID NO:26 modified with a 43 kDa PEG at the C terminus at 10 μ l volume using an infusion pump (Harvard Apparatus). Peptides or vehicle were infused during 2 minutes and the injection needle was kept in place an additional minute after infusion. Five minutes post infusion, rats received an oral dose of 10% charcoal, 5% gum arabic, and 1% carboxymethylcellulose in a volume of 0.8 ml. Five minutes post charcoal, the rats were euthanized by CO₂ inhalation and decapitated. The intestines were dissected out, and the distance the charcoal traveled beyond the pyloric sphincter determined. After the experiment, correct placement of the cannula was verified by injection of Evans Blue and brain section.

[160] At 20 μ g/rat, SEQ ID NO:26 modified with the 43 kDa PEG produced a significant reduction on gastric motility. This effect was comparable to the one produced by

0.5 µg/rat of GLP-1 (7-36) amide peptide (Figure 5). These data demonstrate that a GLP-1 receptor agonist that is linked to a PEG polymer having a molecular weight of greater than 30 kD (i.e., the modified GLP-1 receptor agonist of this invention) is unable to cross the blood brain barrier, unlike the typical GLP-1 agonist. As a result, the modified GLP-1 receptor agonist of this invention can prevent a reduction in gastrointestinal motility, typically associated with a GLP-1 receptor agonist.

[161] Example 9. Pharmaceutical composition – IV and SC formulations

A sterile IV injectable formulation is made from a derivatized polypeptide (e.g., SEQ ID NO: 28 linked to a 43 kD PEG) having equivalent of 4 mg polypeptide content, and 1 liter of sterile saline, using any manufacturing process well known in the art. Higher concentration of derivatized polypeptide may be needed for SC formulation.

[162] All publications and patents mentioned in the above specification are incorporated herein by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by this invention.

What is claimed:

1. A polypeptide selected from the group consisting of SEQ ID NOs: 1, 2, 4-10, 13, 14, and 16-31 and functionally equivalent fragments, derivatives, and variants thereof.
2. The polypeptide of claim 1, wherein said polypeptide is linked to a polyethylene glycol polymer.
3. The polypeptide of claim 2, wherein said polyethylene glycol has a molecular weight of at least 30 kD.
4. The polypeptide of claim 3, wherein said polyethylene glycol is branched.
5. A polynucleotide encoding a polypeptide sequence of claim 1, or a degenerate variant thereof.
6. A vector comprising a polynucleotide of claim 5.
7. A host cell comprising a vector of claim 6.
8. A method for producing a polypeptide comprising:
 - a) culturing the host cell of claim 7 under conditions suitable for the expression of said polypeptide; and
 - b) recovering the polypeptide from the host cell culture.
9. A method for reducing or inhibiting gastrointestinal side effects of a GLP-1 receptor agonist comprising the step of linking a polyethylene glycol polymer to said GLP-1 receptor agonist.
10. The method of claim 9, wherein said polyethylene glycol has a molecular weight of at least 30 kD.
11. The method of claim 10, wherein said polyethylene glycol is branched.
12. The method of claim 9, wherein said GLP-1 receptor agonist is selected from the group consisting of SEQ ID NOs: 1, 2, 4-10, 13, 14, and 16-31 and functionally equivalent fragments, derivatives, and variants thereof.
13. A pharmaceutical composition comprising a therapeutically effective amount of a polypeptide of any one of claims 1 to 4, or functionally equivalent fragments, derivatives, and variants thereof, in combination with a pharmaceutically acceptable carrier.
14. A pharmaceutical composition comprising a therapeutically effective amount of a

polypeptide of any one of claims 1 to 4, or functionally equivalent fragments, derivatives, and variants thereof, in combination with a pharmaceutically acceptable carrier and one or more pharmaceutical agents.

15. The pharmaceutical composition of claim 14, wherein said pharmaceutical agent is selected from the group consisting of PPAR agonists, sulfonylurea drugs, non-sulfonylurea secretagogues, α -glucosidase inhibitors, insulin sensitizers, insulin secretagogues, hepatic glucose output lowering compounds, insulin, anti-obesity agents, HMG CoA reductase inhibitors, nicotinic acid, bile acid sequestrants, fibrin acid derivatives, and anti-hypertensive agents.
16. A composition comprising an effective amount of a polypeptide of any one of claims 1 to 4, or functionally equivalent fragments, derivatives, and variants thereof, in combination with an inert carrier.
17. A method of treating diabetes in a subject comprising the step of administering to a subject in need thereof a therapeutically effective amount of a polypeptide of any one of claims 1 to 4.
18. The method of claim 17, wherein said diabetes is selected from the group consisting of type 1 diabetes, type 2 diabetes, maturity-onset diabetes of the young, latent autoimmune diabetes adult, gestational diabetes, Syndrome X.
19. The method of claim 17, wherein said polypeptide is administered in combination with one or more pharmaceutical agents.
20. The method of claim 19, wherein said pharmaceutical agent is selected from the group consisting of PPAR agonists, sulfonylurea drugs, non-sulfonylurea secretagogues, α -glucosidase inhibitors, insulin sensitizers, insulin secretagogues, hepatic glucose output lowering compounds, insulin, and anti-obesity agents.
21. The method of claim 17, wherein said polypeptide is administered in combination with one or more agents selected from the group consisting of HMG CoA reductase inhibitors, nicotinic acid, bile acid sequestrants, fibrin acid derivatives, and anti-hypertensive agents.
22. The method of any one of claims 19 to 21, wherein the polypeptide and one or more pharmaceutical agents are administered as a single pharmaceutical dosage formulation.
23. Polypeptides according to any one of claims 1 to 4 for the treatment and/or prophylaxis of diabetes.

24. Medicament containing at least one polypeptide according to any one of claims 1 to 4 in combination with at least one pharmaceutically acceptable, pharmaceutically safe carrier or excipient.
25. Use of polypeptides according to claim 1 for manufacturing a medicament for the treatment and/or prophylaxis of diabetes.
26. Medicament according to claim 24 for the treatment and/or prophylaxis of diabetes.

Examples of Polypeptides of the Invention

<u>Peptide</u>	<u>Sequence</u>	<u>SEQ ID NO:</u>
GLP-1 (7-36)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-NH ₂	1
GLP-1 (7-37)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGRG	2
Exendin-4	HGE GTFTSDLSKQMEEEAVRLFIEWLKNGGPS SGAPPPS-NH ₂	3
G27	HSQGTTFTSDYAKYDARRAKEFIAWLVKGR-NH ₂	4
G51	HSQGTTFTSDYAKYDARRAKEFIAWLVKGRG	5
GX	HSQGTTFTSDYARYLDARRAREFIAWLVVRGRG	6
G1	HSQGTTFTSDSKYLDSSRRAQDFVQWLVVKGR-NH ₂	7
G5	HSQGTTFTSDSKYLEGQAAKEFIAWLVKGR-NH ₂	8
G55	HSQGTTFTSDYARYLDARRAKEFIAWLVKGR-NH ₂	9
G56	HSQGTTFTSDYAAYL DARRAKEFIAWLVKGR-NH ₂	10

Figure 1

Examples of Modified* Polypeptides of the Invention

<u>Peptide</u>	<u>Sequence</u>	<u>SEQ ID NO:</u>
G73	HSQGTTFTSDYAKYLDARRAKEFICWLVKGRG	13
G74	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>C</u> RG	14
G76	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>G</u> CG	16
G77	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>GRC</u>	17
G78	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>GRGC</u>	18
G79	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>GRGS</u> C	19
G80	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>GRGP</u> SC	20
G81	HSQGTTFTSDYAKYLDARRAKEFIAWLVK <u>GRGG</u> SGSC	21
G82	HSQGTTFTSDYARYLDARRAKEFIAWLVK <u>GRGGG</u> SC	22
G83	HSQGTTFTSDYARYLDARRAREFIKWLVRGRG	23
G84	HSQGTTFTSDYARYLDARRAREFIAWLVK <u>GR</u> G	24
G85	HSQGTTFTSDYARYLDARRAREFIAWLVRGRGK	25
G185	HSQGTTFTSDYARYLDARRAREFIKWLVRGR <u>C</u>	26
GLP-1	HAEGLTFTSDVSSYLEQAAKEFIAWLVK <u>GRGC</u>	27
GLP-1	HAEGLTFTSDVSSYLEQAAKEFICWLVK <u>GRGG</u>	28
GLP-1	HAEGLTFTSDVSSYLEQAAKEFIAWLVK <u>GRGG</u>	29
GLP-1	HAEGLTFTSDVSSYLEQAAKEFIAWLVK <u>GRCG</u>	30
Exendin-4	HGEGLTFTSDLSKQMEEAVRLIEWLKNGGPSSGAPPSC	31

*PEG attached to unique cysteine or lysine using the respective cysteine or lysine chemistry.
Underline amino acid illustrates position PEG attachment

Figure 2

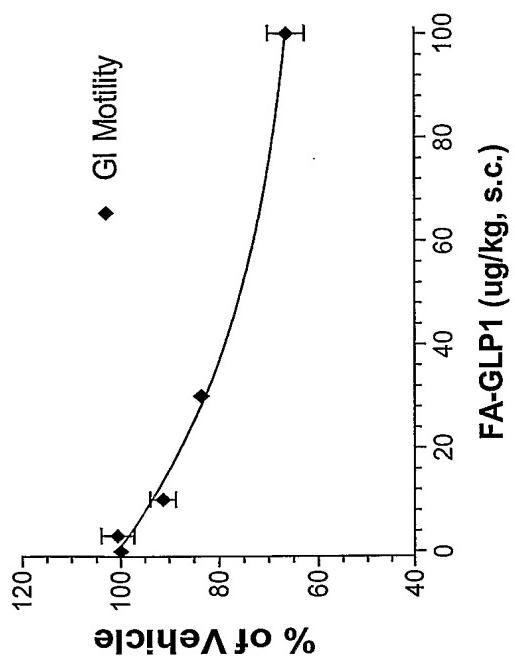


Figure 3a

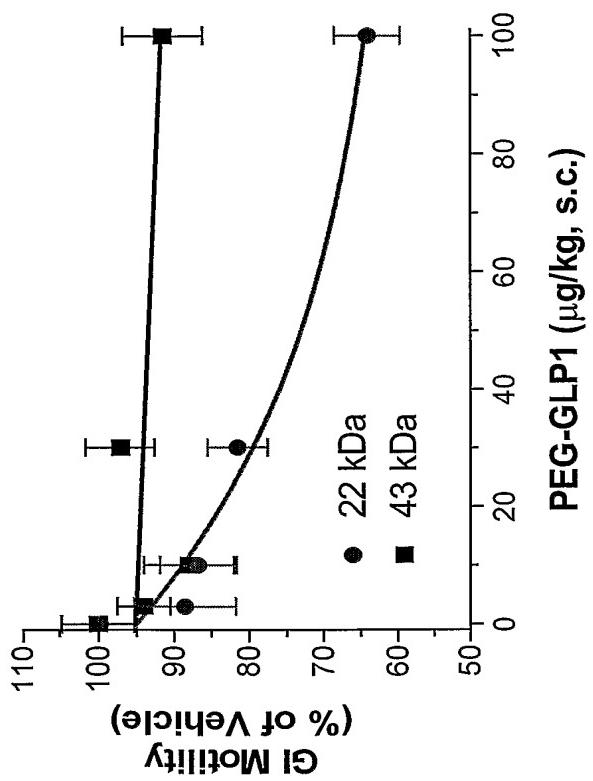


Figure 3b

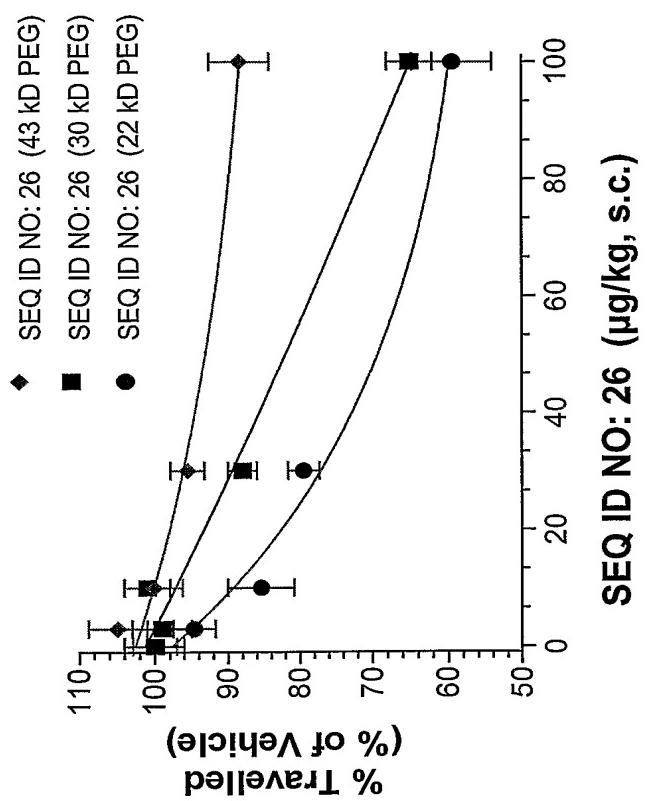


Figure 4

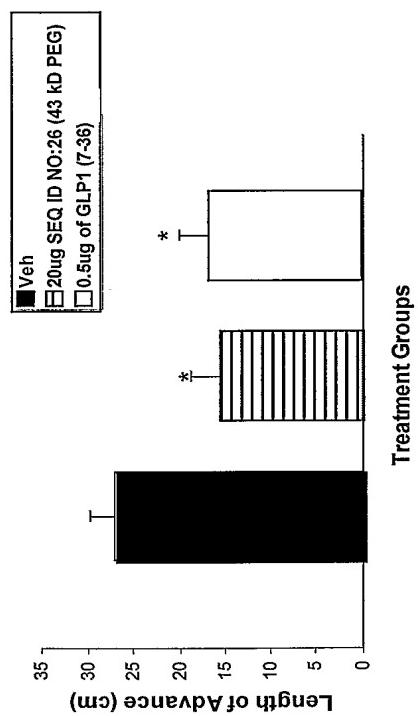


Figure 5

SEQUENCE LISTING

<110> Bayer Pharmaceuticals Corporation
Pan, Clark
Whelan, James
<120> Modified GLP-1 Receptor Agonists and Their Pharmacological Methods of Use
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<150> US 60/408,696
<151> 2002-09-16
<150> US 60/439,369
<151> 2003-01-09
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